

INHIBIN MODULATION OF CELL GROWTH

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The present invention relates generally to a method of modulating cell growth and more particularly, to a method of modulating prostate cell growth. Even more particularly, the present invention provides a method of treating prostate cancer by inhibiting division of malignant prostate cells.

The present invention also relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer.

The bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Disorders of the prostate gland are of particular concern in ageing men. Figures suggest that approximately one in four males above the age of 55 will suffer from a prostate disease in some form. The incidence in Australia of prostatic cancer is high and similarly prevalent rates occur in most communities. This represents a significant cost to health care systems and decreases the quality of life of men suffering from this disorder.

Inhibins are gonadal derived hormones which have a negative feedback action on the release of pituitary follicle stimulating hormone (FSH). They consist of an  $\alpha$  and either a  $\beta_A$  or  $\beta_B$  subunit linked by disulphide bonds (Burger *et al.*, 1996). Inhibin A is formed by the dimerisation of  $\alpha$  and  $\beta_A$  subunits; inhibin B from dimerisation of  $\alpha$  and  $\beta_B$  subunits.

The  $\alpha$ -inhibin subunit is synthesised in precursor forms consisting of pre, pro,  $\alpha N$  and  $\alpha C$  components. The precursor  $\alpha$  and  $\beta$  subunits link to form a 105 kD bioactive inhibin,

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which forms 31-34 kD inhibin  $\alpha$ C- $\beta$  after postranslational modification (cleavage of the pre, pro and  $\alpha$ N regions from the  $\alpha$ -subunit and pro from the  $\beta$  subunit) (Robertson *et al.*, 1994). Inhibin B is considered to be the physiologically important form of inhibin which regulates FSH release in men (Illingworth *et al.*, 1996). Dimerisation of two  $\beta$  subunits results in the formation of activin. Three dimeric forms of activins have been published, Activin A ( $\beta_A \beta_A$ ), Activin B ( $\beta_B \beta_B$ ) and Activin AB ( $\beta_A \beta_B$ ). In contrast to the inhibins, the activins stimulate pituitary FSH (Ling *et al.*, 1986).

The inhibin  $\beta$  subunits show approximately 30% homology with the  $\beta$  subunits of TGF  $\beta$  and thus, inhibins are members of the TGS  $\beta$  superfamily of growth and differentiation factors (Massague, 1990). In accordance with this classification, the inhibins have been shown to have a wide range of effects, in addition to the regulation of FSH. In erythroid, immune and endocrine tissues, both proliferative and antiproliferative actions of inhibin has been described (Mather *et al.*, 1990; Hedger *et al.*, 1989; Kaipia *et al.*, 1994). Activin A has also been reported to induce apoptosis (Nishihara *et al.*, 1993). In many instances, the actions of inhibins can be antagonised by activins (Hseuh *et al.*, 1987). The actions of activins are mediated through specific serine/threonine kinase receptors (Matthews *et al.*, 1991). No specific receptors for inhibins have been isolated to date. In addition to receptors for activin, there are binding proteins for activins which include follistatins (Nakamura *et al.*, 1990). Follistatins have no structural homology to inhibins or activins but can bind strongly to activins and, in doing so, suppress or neutralise their bioactivity (Mather *et al.*, 1993). Two mRNA species have been identified for follistatin which arise from alternate splicing, and result in two proteins denoted FS288 and FS315. FS288 has been demonstrated to be membrane associated, while FS315 is a secreted protein (Michel *et al.*, 1990).

Franchimont (1982) showed that seminal plasma is a source of inhibin, as plasma from normal men significantly suppressed serum FSH when administered to castrate rates. More recently, it has been reported that the rat ventral prostate gland itself is a site of synthesis of inhibin and related proteins (Risbridger *et al.*, 1996).

Understanding the cellular localisation and expression of inhibin in prostate tissue from men with and without carcinoma of the prostate is required to determine the role of inhibin in prostate cancer. In work leading up to the present invention, the inventors discovered that in tissues from men with benign prostatic hyperplasia, basal cell

5 hyperplasia or in non-malignant regions of specimens from men with prostate cancer, inhibin  $\alpha$ -subunit mRNA and protein expression were observed. In contrast, in malignant regions of tissue from men with advanced stage prostate cancer the localisation and expression of inhibin  $\alpha$ -subunit was down regulated in that mRNA and protein were not detectable in poorly differentiated tumour cells.

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Accordingly, one aspect of the present invention relates to a method of modulating cell growth in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding inhibin.

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Reference hereinafter to "inhibin" should be read as including reference to all forms of inhibin and fragments thereof or derivatives, homologues, analogues, mutants and variants thereof including all subunit polypeptides thereof including by way of example any protein encoded by the  $\alpha$  or  $\beta$  subunit gene, the monomeric  $\alpha$ -subunit polypeptide, the subunit precursor polypeptides pre, pro  $\alpha$ N and  $\alpha$ C, the monomeric  $\beta$  subunit polypeptide, the dimeric  $\alpha\beta$  polypeptide (for example  $\alpha\beta_A$ ,  $\alpha\beta_B$ ,  $\alpha\beta_C$ ,  $\alpha\beta_D$ , and  $\alpha\beta_E$ ) the dimeric precursor  $\alpha$ C- $\beta$  polypeptide and including, but not limited to, derivatives, homologues, analogues, mutants and variants thereof.

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25 Preferably, said inhibin is the  $\alpha$ -subunit polypeptide ( $\alpha$ -inhibin) or fragment thereof or derivative, homologue, analogue, mutants and variants thereof. Reference to  $\alpha$ -inhibin, hereinafter, is not intended to be limiting and should be read as including reference to all forms of  $\alpha$ -inhibin including any protein encoded by the  $\alpha$ -subunit gene, all subunit polypeptides thereof including by way of example the monomeric subunit precursor

30 polypeptides pre, pro  $\alpha$ N and  $\alpha$ C, and including, but not limited to, derivatives,

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homologues, analogues, mutants and variants thereof.

More particularly, the present invention relates to a method of modulating cell growth in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding  $\alpha$ -inhibin.

The term "mammal" includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, rabbits, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably, the mammal is a human.

The term "modulating" means up-regulating or down-regulating. Accordingly, although the preferred method is to increase the expression of a genetic sequence encoding  $\alpha$ -inhibin, the reduction of the expression of a genetic sequence encoding  $\alpha$ -inhibin expression may also be desired under certain circumstances.

The term "expression" refers to the synthesis of a polypeptide utilising the mechanisms of transcription and translation of a nucleic acid molecule.

Although not intending to limit the present invention to any one mode of action, modulation of the expression of a genetic sequence encoding  $\alpha$ -inhibin by the administration of an agent to a mammal can be achieved via one of several techniques including but in no way limited to:

- (i) introduction of a nucleic acid molecule encoding  $\alpha$ -inhibin or a derivative thereof to modulate the capacity of that cell to synthesize  $\alpha$ -inhibin;

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(ii) introduction into a cell of a proteinaceous or non-proteinaceous molecule which modulates promoter operation of a gene;

5 (iii) introduction into a cell of a proteinaceous or non-proteinaceous molecule which modulates transcriptional and/or translational regulation of a gene.

Said gene may be an  $\alpha$ -inhibin gene or some other gene which directly or indirectly regulates the expression of an  $\alpha$ -inhibin gene.

10 Preferably, expression of a genetic sequence encoding  $\alpha$ -inhibin expression is modulated in prostate cells and even more preferably the prostate cells are malignant.

According to this preferred aspect of the present invention there is provided a method of modulating malignant prostate cell growth in a mammal said method comprising  
15 administering to said mammal an effective amount of agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding  $\alpha$ -inhibin.

Although not intending to limit the present invention to any one theory or mode of action the basal epithelial cells of the prostate gland are the predominant site of the expression of  
20 the  $\alpha$ -inhibin gene. The synthesis and production of the  $\alpha$ -inhibin subunit protein in prostatic basal epithelium correlates with data demonstrating that the  $\beta$ -inhibin subunit proteins are localised in these cells. Since both inhibin  $\alpha$  and  $\beta$  subunits are expressed in the same cells the tissues have the ability to produce  $\alpha\beta$  dimeric inhibin protein. The observation that  $\alpha$ -inhibin mRNA and protein is observed in epithelial cells in benign  
25 prostate tissues and basal cell hyperplasia but not in poorly differentiated malignant prostate epithelial cells is consistent with tumour suppressive activity of  $\alpha$ -inhibin in the prostate gland.

Accordingly, in a preferred embodiment said expression of a genetic sequence encoding  $\alpha$ -  
30 inhibin is up-regulated.

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In a particularly preferred embodiment, up-regulation of a genetic sequence encoding  $\alpha$ -inhibin inhibits cell growth.

According to this most preferred embodiment, the present invention relates to a method of  
5 inhibiting malignant prostate cell growth in a mammal said method comprising  
administering to said mammal an effective amount of an agent for a time and under  
conditions sufficient to up-regulate the expression of a genetic sequence encoding  $\alpha$ -  
inhibin.

10 The modulation of cell growth in a mammal via the modulation of the expression of a  
genetic sequence encoding inhibin can also be achieved by the administration of inhibin to  
said mammal.

Accordingly, another aspect of the present invention relates to a method of modulating cell  
15 growth in a mammal said method comprising administering to said mammal an effective  
amount of inhibin.

Preferably, said cells are prostate and even more preferably said prostate cells are  
malignant.

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Most preferably cell growth is inhibited.

Accordingly, in a preferred embodiment the present invention relates to a method of  
inhibiting malignant prostate cell growth in a mammal said method comprising  
25 administering to said mammal an effective amount of inhibin.

Yet more preferably, said inhibin is  $\alpha$ -inhibin.

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It has been observed that there is a high degree of homology between inhibins from different mammalian species. Thus the inhibin used may be derived from any origin including human, primate, bovine, ovine, porcine or other mammalian or animal species. Preferably, the inhibin is recombinant human inhibin.

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The term "inhibin" used herein include fragments, said fragments having the functional activity of inhibin and including but not limited to homologues, analogues, mutants, variants and derivatives thereof. This includes homologues, analogues, mutants, variants and derivatives derived from natural or recombinant sources including fusion proteins.

10 Reference to "inhibin" should also be understood to encompass inhibin agonists.

The homologues, analogues, mutants, variants and derivatives may be derived from insertion, deletion or substitution of amino acids in the inhibin. Amino acid insertional derivatives of inhibin used in the present invention include amino and/or carboxylic

15 terminal fusions as well as intra-sequence insertions of single or multiple amino acids.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence.

20 Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins. Typical substitutions are those made in accordance with Table 1:

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TABLE 1

## Suitable residues for amino acid substitutions

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
	Ala	Ser
5	Arg	Lys
	Asn	Gln; His
	Asp	Glu
	Cys	Ser
	Gln	Asn
10	Glu	Ala
	Gly	Pro
	His	Asn; Gln
	Ile	Leu; Val
	Leu	Ile; Val
15	Lys	Arg; Gln; Glu
	Met	Leu; Ile
	Phe	Met; Leu; Tyr
	Ser	Thr
	Thr	Ser
20	Trp	Tyr
	Tyr	Trp; Phe
	Val	Ile; Leu

The inhibin of the present invention may be in monomeric or multimeric form meaning  
 25 that two or more molecules are associated together. Where the same inhibin molecules are  
 associated together, the complex is a homomultimer. An example of a homomultimer is a  
 homodimer. Where at least one inhibin molecule is associated with at least one non-  
 inhibin molecule, then the complex is a heteromultimer such as a heterodimer.

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Inhibin suitable for use in the present invention may be the inhibin glycoprotein which has a molecular weight of 31 kD in its dimeric form and is made up of a 20 kD  $\alpha$ -subunit and a 14 kD  $\beta$ -subunit. Preferably the inhibin used in the present invention may be that described in Robertson *et al.*, (1985) or Forage *et al.*, or similar.

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The inhibin suitable for use in the method of treatment aspect of the present invention is not related to the "inhibin" described in WO93/25224 which is a non-glycosylated protein occurring in two forms having molecular weight of 10.5 kD and 16 kD.

- 10 Although the preferred method is to down-regulate cell growth, in particular malignant prostate cell growth, the up-regulation of cell growth may be desired under certain circumstances.

- Accordingly, in another aspect the present invention provides a method of modulating cell growth in a mammal said method comprising administering a mammalian cell growth modulating effective amount of an inhibin antagonist to said cells.

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Preferably, said cells are prostate cells.

- 20 Accordingly, in this preferred aspect the present invention provides a method of modulating growth of mammalian prostatic cells comprising administering a prostatic cell growth modulating amount of an inhibin antagonist to said cells.

The term "modulating" has the same meaning as given above.

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- The antagonists may be any compound capable of blocking, inhibiting, or otherwise preventing inhibin from carrying out its normal biological functions in prostate cells or tissue. Antagonists include monoclonal antibodies specific for inhibin, or parts of inhibin, and antisense nucleic acids which prevent transcription or translation of inhibin genes or mRNA in mammalian cells. Antagonists also include analogues of inhibin which bind the

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inhibin receptors and thereby prevent inhibin from performing its normal biological functions in the prostate. Antagonists in the form of analogues may include those analogues described above. Antisense sequences based on the nucleotide sequences of inhibin disclosed in US Patent 4,740,587 and Forage *et al* (1986) are also contemplated.

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The agent, inhibin, or inhibin antagonist used may also be linked to a targeting means, such as a monoclonal antibody, which provides specific delivery of the agent, inhibin or antagonist to the cells.

- 10 In a preferred embodiment of the present invention, the agent, inhibin or inhibin antagonist used in the method is linked to an antibody specific for the prostate to enable specific delivery to this organ.

Administration of the agent, inhibin, or inhibin antagonists, in the form of a

- 15 pharmaceutical composition, may be performed by any convenient means. The agent, inhibin or inhibin antagonists of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the inhibin or inhibin antagonist chosen. A broad range of doses may be applicable.

- 20 Considering a patient, for example, from about 0.1 mg to about 1 mg of inhibin or antagonist may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

- 25 The inhibin or part thereof or antagonist may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of inhibin, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid

- 30 addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as

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salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch  
5 or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

The tumour suppressing action of inhibins may be mediated through specific receptor complexes similar to those described for TGF- $\beta$  and activin. The existence of receptors  
10 for inhibins have not been identified although there is a body of indirect evidence to suggest that such receptors exist (Woodruff *et al.*, 1992; Krummen *et al.*, 1994).

A further aspect of the present invention relates to the use of the invention in relation to human disease conditions. For example, the present invention is particularly useful, but in  
15 no way limited to use in inhibiting growth of malignant prostate cells.

Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic  
20 sequence encoding inhibin.

Preferably, said inhibin is  $\alpha$ -inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are  
25 malignant.

Most preferably expression of a genetic sequence encoding  $\alpha$ -inhibin is up-regulated.

Yet even more preferably cell growth is inhibited.

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Accordingly, in a preferred embodiment the present invention relates to a method of treating malignant prostate cells in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to up-regulate the expression of a genetic sequence encoding  $\alpha$ -inhibin.

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The treatment of a mammal by the administration of an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a genetic sequence encoding inhibin and thereby regulating cell growth can also be achieved by the administration of inhibin to said mammal.

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Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of inhibin.

15 Preferably, said inhibin is  $\alpha$ -inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

20 Most preferably cell growth is inhibited.

Accordingly, in a preferred embodiment the present invention relates to a method of treating malignant prostate cells in a mammal said method comprising administering to said mammal an effective amount of  $\alpha$ -inhibin.

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In yet another aspect the present invention relates to the use of an agent capable of modulating the expression of a genetic sequence encoding inhibin in the manufacture of a medicament for the modulation of cell growth in a mammal.

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Preferably said cells are prostate and even more preferably said prostate cells are malignant.

Most preferably cell growth is inhibited.

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Yet more preferably said inhibin is  $\alpha$ -inhibin.

Most preferably, expression of a genetic sequence encoding  $\alpha$ -inhibin is up-regulated.

- 10 Yet another aspect of the present invention relates to the use of inhibin in the manufacture of a medicament for the modulation of cell growth in a mammal.

Preferably said cells are prostate and even more preferably said prostate cells are malignant.

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Most preferably cell growth is inhibited.

Yet more preferably said inhibin is  $\alpha$ -inhibin.

- 20 A related aspect of the present invention relates to agents for use in modulating the expression of a genetic sequence encoding inhibin wherein modulating expression of said genetic sequence regulates cell growth.

Preferably said inhibin is  $\alpha$ -inhibin.

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Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Most preferably the expression of a genetic sequence encoding  $\alpha$ -inhibin is up-regulated.

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Yet even more preferably cell growth is inhibited.

In yet another related aspect the present invention relates to inhibin for use in regulating cell growth.

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Preferably, said inhibin is  $\alpha$ -inhibin.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

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Yet even more preferably cell growth is inhibited.

In a related aspect of the present invention the mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment of a prostate disorder

15 or a potential prostate disorder.

The tumour suppressive function of  $\alpha$ -inhibin protein is predicated on the observation that  $\alpha$ -inhibin mRNA and protein is present in the prostate basal epithelial cells of patients with benign prostate disease and in said epithelial cells located in non-malignant regions of prostatic tissue from patients exhibiting prostate cancer but not in the malignant regions of said cancerous prostates.

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Accordingly, another aspect of the present invention relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer, said method comprising screening for the down-regulation of the inhibin protein levels and/or gene expression in said mammal, wherein the down-regulation of the inhibin protein levels and/or gene expression is indicative of said mammal being predisposed to prostate cancer or having already developed prostate cancer.

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Reference to "down-regulation" should be understood to include reference to the complete absence or total loss of protein and/or gene expression.

"Inhibin" has the same meaning as given above and should therefore be understood to include any protein, or fragment thereof, encoded by the  $\alpha$  or  $\beta$  subunit gene whether existing as a monomer, multimer or fusion protein. Examples of a multimer include the  $\alpha\beta$  heterodimer or a heterodimer comprising any protein encoded by the  $\alpha$ -subunit gene in association with any other protein.

10 Preferably said inhibin is  $\alpha$ -inhibin.

" $\alpha$ -inhibin" also has the same meaning as given above. Accordingly, reference to  $\alpha$ -inhibin includes, by way of example, reference to any protein, or fragment thereof, encoded by the  $\alpha$ -subunit gene, whether existing as a monomer, multimer or fusion protein. Proteins encoded by the  $\alpha$ -subunit gene include, for example, pre-pro- $\alpha$ N- $\alpha$ C, pro- $\alpha$ C and the cleavage products  $\alpha$ N,  $\alpha$ C, pre-pro or isoforms thereof.

The  $\alpha$ -inhibin proteins which are detectable in the prostates from patients diagnosed with benign prostate hyperplasia or in the non-malignant regions of prostate may comprise, for example,  $\alpha$ N and/or  $\alpha$ C regions. The present invention is exemplified, but not limited in any way, by reference to detection of  $\alpha$ -inhibin levels via the detection of the  $\alpha$ N or  $\alpha$ C regions of the  $\alpha$ -inhibin protein.  $\alpha$ -inhibin proteins comprising  $\alpha$ N and/or  $\alpha$ C regions are also referred to as precursor  $\alpha$ -subunit proteins. The  $\alpha$ N and/or  $\alpha$ C regions of precursor  $\alpha$ -subunit proteins are found to exist either as part of an existing precursor  $\alpha$ -subunit protein or in isolation, for example, following cleavage of said region from a precursor  $\alpha$ -subunit protein. Precursor  $\alpha$ -subunit proteins exist in many forms including, but not limited to, the forms pre- pro-  $\alpha$ N -  $\alpha$ C and pro- $\alpha$ C. According to this embodiment of the present invention, detection of  $\alpha$ -inhibin proteins, including precursor  $\alpha$ -subunit proteins, includes the detection of the  $\alpha$ N and/or  $\alpha$ C regions both in isolation, and as part of one or more of the various forms of precursor  $\alpha$ -subunit protein. The  $\alpha$ C and  $\alpha$ N regions can be detected, either in isolation or as part of a precursor  $\alpha$ -subunit protein, using, for example, the polyclonal

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antibodies # $\alpha$ C41 and  $\alpha$ N320, respectively.

Accordingly, a preferred embodiment of the present invention relates to a method of screening for a mammal having prostate cancer, said method comprising screening for the  
5 down-regulation of  $\alpha$ C or isoform thereof in said individual, wherein the down-regulation of the  $\alpha$ C or isoform thereof is indicative of prostate cancer.

In another preferred embodiment, the present invention relates to a method of screening for a mammal having prostate cancer said method comprising screening for the down-regulation of  
10  $\alpha$ N or isoform thereof in said individual, wherein the down-regulation of  $\alpha$ N or isoform thereof is indicative of prostate cancer.

In yet another preferred embodiment, the present invention relates to a method of screening for a mammal having prostate cancer said method comprising screening for the  
15 down-regulation of  $\alpha$ -subunit gene expression in said individual, wherein the down-regulation of  $\alpha$ -subunit gene expression is indicative of prostate cancer.

Without limiting the invention to any one theory or mode of action, in the pre-malignant prostate analysis of  $\alpha$ C and/or  $\alpha$ N, or isoform thereof, expression reveals disruption of the  
20 basement membrane and basal cells when compared to a non-cancerous prostate.

Accordingly, a related embodiment of the present invention relates to a method of screening for a mammal having a predisposition to prostate cancer, said method comprising screening for  $\alpha$ -subunit gene expression in said individual, wherein  $\alpha$ -subunit  
25 gene expression reveals disruption of the basement membrane, said disruption indicating a predisposition to prostate cancer.

Although not intending to limit the present invention to any one mode or theory of action, the absence of  $\alpha$ -inhibin protein expression in the malignant prostate results in the inability  
30 of  $\beta$  subunit protein monomers to form inhibin  $\alpha\beta$  dimers. Since activin is formed by the dimerisation of two  $\beta$  subunits, modulation of activin levels in the prostate provides an

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additional and/or alternative indicator of malignancy.

Accordingly, another aspect of the present invention relates to a method of screening for a mammal having prostate cancer or a predisposition to prostate cancer, said method  
5 comprising screening for the modulation of activin protein levels in said mammal, wherein the modulation of activin protein levels is indicative of said mammal being predisposed to prostate cancer or having already developed prostate cancer.

Screening of inhibin, and/or activin protein levels or gene expression in a mammal can be  
10 achieved via one of several techniques including but in no way limited to:

- (i) *in situ* hybridisation of prostate tissues with probes detecting inhibin  $\alpha\beta$  dimers or monomers thereof.
- 15 (ii) immunohistochemistry of prostate tissues utilising antibody directed any region of to the  $\alpha$  monomeric subunit, the  $\beta$  monomeric subunit and/or the  $\alpha$ C or  $\alpha$ N or isoform of the  $\alpha$  monomeric subunit.
- (iii) quantitative measurement of the activin and/or inhibin protein in prostate tissue.
- 20 (iv) analysis of  $\alpha$  or  $\beta$  subunit mRNA expression.
- (v) screening of blood to detect  $\alpha\beta$  dimers or monomers thereof, the  $\alpha$  monomeric subunit, the  $\beta$  monomeric subunit, and/or  $\alpha$ C or  $\alpha$ N or isoform of the  $\alpha$  monomeric  
25 subunit.

This method is particularly important for prostate cancer.

Preferably, the mammal is human.

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In one particularly preferred method, the target inhibin molecules in the biological sample are exposed to a specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with an antibody.

- 5 Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by  
10 its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

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In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase,  
20 glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the  
25 chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated,  
30 usually spectrophotometrically, to give an indication of the amount of hapten which was

present in the sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

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In another aspect the present invention relates to a pharmaceutical composition comprising an agent capable of modulating expression of a genetic sequence encoding inhibin thereby regulating cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

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Preferably, said inhibin is  $\alpha$ -inhibin.

According to this preferred embodiment the present invention relates to a pharmaceutical composition comprising an agent capable of regulating expression of a genetic sequence encoding  $\alpha$ -inhibin expression thereby regulating cell growth.

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In a particularly preferred embodiment expression of a genetic sequence encoding  $\alpha$ -inhibin is up-regulated.

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In yet another most preferred embodiment up-regulation of expression of a genetic sequence encoding  $\alpha$ -inhibin inhibits cell growth.

Accordingly the present invention relates to a pharmaceutical composition comprising an agent capable of up-regulating expression of a genetic sequence encoding  $\alpha$ -inhibin thereby inhibiting cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

Another aspect of the present invention relates to a pharmaceutical composition comprising inhibin capable of regulating cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said inhibin is  $\alpha$ -inhibin.

In a particularly preferred embodiment  $\alpha$ -inhibin inhibits cell growth.

Accordingly the present invention relates to a pharmaceutical composition comprising  $\alpha$ -inhibin capable of inhibiting cell growth and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said cells are prostate cells and even more preferably said prostate cells are malignant.

These components are referred to as the active ingredients.

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The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed

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in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like.

- 5 Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the
- 10 present invention are prepared so that an oral dosage unit form contains between about 0.1  $\mu$ g and 2000 mg of active compound.

- The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as
- 15 dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other
- 20 materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be
- 25 pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention is further described by the following non-limiting figures and/or examples.

In the Figures:

5

**Figure 1** is a photographic representation of analysis of  $\alpha$ -subunit mRNA by RT-PCR.

**Figure 2a** is a graphical representation of radioactive profiles of inhibin tracer incubated in prostate cytosol for 2 days.

10

**Figure 2b** is a graphical representation of radioactive profile of activin tracer incubated in prostate cytosol.

**Figure 3** is a graphical representation of logit plots of  $B/B_0$  recombinant human activin standard (Has3) and rat prostate cytosol.

15

**Figure 4** is a photographic representation of the localisation of inhibin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunit proteins to BPH prostate tissue.

(A-C): Prostate biopsy tissue (A) did not exhibit any inhibin  $\alpha$  immunoreactivity in the glandular epithelium or the stroma. In contrast, using the same  $\alpha$ -subunit antibody, specific immunoreactivity was localised to the stromal cells of a benign mucinous cystadenoma of the ovary (C). Control prostate tissue incubated with anti-mouse IgG did not detect any positive immunoreactivity (B).

20

(D-F): Prostate biopsy tissues (D:x20 magnification; E:x40 magnification) stained positively using the  $\beta_A$  subunit antibody, and specific immunoreactivity was localised to the glandular epithelium. Note that there was variable staining within the glandular epithelium itself, as indicated by arrows. Control tissue incubated with normal rabbit serum did not show any positive immunoreactivity (F).

25

(G-I): Prostate biopsy tissue immunostained with the  $\beta_B$  subunit antibody, showed weak immunoreactivity which was localised to the glandular epithelium (G & H). No specific localisation was recorded in control tissue incubated with the anti-mouse IgG antibody (I).

30

(Scale bar in A represent 20 microns and is applicable to A, B, C, D, F, G and I. Scale bar in E represent 20 microns and is applicable to E, G and H).

**Figure 5** is a photographic representation of RT-PCR and Southern analysis of inhibin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits, the putative activin  $\beta_C$  subunit, the activin type II receptor (ActRII) and FS288 and FS315 mRNA expression in human BPH biopsy samples. mRNA extracted from two groups of human biopsy samples were analysed by RT-PCR and Southern analysis. Total RNA from adult rat testes (t) and adult rat prostate(p) were used as positive controls, water (w) was used as a negative control. The size of the RT-PCR products was confirmed using pGEM DNA molecular weight markers (Promega Biotec, Madison, USA) (m). The expression of the activin receptor, ActRII (A), inhibin  $\beta_A$  subunit (B), and the putative activin  $\beta_C$  subunit (C) were determined in patients a-e (Lanes a-e). Follistatin (D), inhibin  $\beta_B$  (E) and  $\alpha$  (F) subunit mRNA expression was determined in patients f-j (Lanes f-j).

15

**Figure 6** is a photographic representation of the localisation of inhibin  $\alpha_C$  and  $\alpha_N$  subunit proteins and  $\alpha$ mRNA to benign prostatic hyperplasia tissue. The basal cells in the prostatic epithelium of the benign prostate biopsy tissue stained positively using the cytokeratin marker antibody (A). Control prostate tissue incubated with mouse IgG did not detect any specific localisation (B). Specific immunoreactivity for inhibin  $\alpha_C$  protein was detected in basal cells of the prostate epithelium (C). Control tissue incubated with sheep IgG did not show any positive immunoreactivity (D). Both basal cells and secretory epithelium displayed immunoreactivity for inhibin  $\alpha_N$  protein (E). No specific localisation was recorded in the control tissue incubated with sheep IgG (F).  $\alpha$ -inhibin mRNA was expressed in epithelial basal cells in the benign prostate (G) and in one patient, in both basal and secretory epithelial cells (H - note the section has been counterstained). The localisation was detected with the sense probe (I and J).

25

- Figure 7** is a photographic representation of the localisation of inhibin  $\alpha$ C and  $\alpha$ N subunit proteins and  $\alpha$  mRNA to patients with basal cell hyperplasia. Cytokeratin specific antibody identified areas of basal cell hyperplasia in benign prostate tissue (A). Incubation of the control section with mouse IgG showed no specific immunoreactivity (B). The same regions displayed positive immunoreactivity for both  $\alpha$ C and  $\alpha$ N inhibin protein (C and E, respectively). Control sections incubated with sheep IgG displayed no positive localisation (D and F, respectively).  $\alpha$ -inhibin mRNA was positively expressed in basal cell hyperplasia (G). No specific localisation was detected with the sense probe (H).
- 10 **Figure 8** is a photographic representation of the localisation of inhibin  $\alpha$ C and  $\alpha$ N subunit proteins and  $\alpha$ -inhibin mRNA to non-malignant and malignant regions of prostate tissue from patients with high grade prostate cancer. Inhibin  $\alpha$ C protein was localised to the basal epithelial cells in the non-malignant region (A) of the prostate biopsy. The adjacent tumour cells displayed no positive immunoreactivity (B). Specific localisation of  $\alpha$ N
- 15 protein was observed in the secretory epithelium of the non-malignant region (C); the adjacent tumour tissue displayed no positive staining (D). A control section was incubated with sheep IgG and displayed no specific immunoreactivity (E and F).  $\alpha$ -inhibin mRNA was expressed in basal epithelial cells in the non-malignant region (G). The adjacent malignant region showed no positive localisation (H). The control section incubated with
- 20 the sense probe displayed no staining (I and J).

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**EXAMPLE 1**  
**DETECTION OF INHIBIN SUBUNIT AND RECEPTOR**  
**GENE EXPRESSION BY RT-PCR**

5 This Example indicates that inhibin and activin genes as well as activin receptor genes are expressed in the prostate.

**Methods:**

***RNA Extraction***

10 Total cellular RNA was extracted for adult rat prostates according to Chomczynsky and Sacchi (1987 Anal Biochem 162: 156-158) method, and checked for integrity by visualisation of 18S and 28S ribosomal RNA bands following electrophoresis on a 1% formaldehyde/agarose gel.

15 ***Oligonucleotide Primers***

The oligonucleotide sequences for the reverse transcription-polymerase chain reaction (RT-PCR) were taken from van den Eijnden-van Raaij *et al* (1992 Dev biol 154: 356-365) and were as follows. The sequence of the downstream primer for inhibin  $\alpha$  was 5' AGC CCA GCT CCT GGA AGG AGA T 3' [SEQ ID NO:1] and the upstream primer was 5' TCA GCC CAG CTG TGG TTC CAC A 3' [SEQ ID NO:2]. For these subunits an intron is absent. Predicted fragment sizes were  $\alpha$ -subunit 444bp.

The oligonucleotide primers for the activin receptors type II and IIB were designed from the rat sequence data available on Genebank: the sequence for the downstream primer of ActR-II was 5' GGA ATT CGC ACC AAT GAA CTG 3' [SEQ ID NO:3] and the upstream primer was 5' CGG GAT CCA ACT GCT ATG ACA GG 3' [SEQ ID NO:4]. The internal primer used for the southern detection was as follows 5' TAG GAC AAT GTG GCT TCG GGT GG 3' [SEQ ID NO:5]. These primers span the extracellular and transmembrane regions of the gene and the predicted fragment size is 510 bp. The ActR-IIb primers are as follows,  
25 downstream 5' AGC CAG CAC CGC GGT GAG 3' [SEQ ID NO:6] and upstream 5' GTG GCT GTG AAG ATC TCC 3' [SEQ ID NO:7]. The internal primer used for the southern  
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analysis is as follows 5' TGG CTC ATC ACA GCC TT 3' [SEQ ID NO:8]. These primers span the serine kinase domain and the predicted product size is 366 bp.

### ***Reverse transcription***

- 5 Reverse transcription was carried out using 0.5 µg total RNA mixed with 4U of AMV reverse transcriptase (Promega Biotec, Madison, WI), 20U of RNasin (Promega Biotec, Madison, WI) 1mM dNTP, 1mM MgCl<sub>2</sub>, 25pmol of the appropriate downstream primer in PCR buffer (Biotec International, Ltd, WA, Australia) to a final volume of 20µl. The solution was incubated at 42°C for 2 hours, then heated to 95°C for 5 min, and cooled
- 10 rapidly on ice.

### ***Polymerase Chain Reaction***

- The PCR was performed in an automatic DNA thermal cycler (Corbett Research Mort Lake Australia) as previously described by Saiki *et al.*, (1988 Science 239: 487-491). Briefly 5µl
- 15 of the RT mixture was added to 0.2mM dNTP, 25pmol of the appropriate upstream primer and 2U of the thermostable *Tth* DNA polymerase (Biotec International Ltd, WA, Australia) in the PCR buffer (Biotec International Ltd, WA, Australia) in a final volume of 20µl. Denaturation was at 95°C for 30 sec, annealing at 56°C for α-subunit, 55°C Act RII and 46°C for ActRIIB for 30 sec, and extension at 72°C for 1 min for a 40 cycle program. The
- 20 products were then analysed by agarose gel electrophoresis in IX TAE.

### ***Results***

- The results show that the inhibin α-subunit gene was detected in prostate tissues from rat tissues from day 10-170 days old by RT-PCR (Figure 1). The activin receptor types II was
- 25 also detected in these tissues. These data suggest that inhibin and activins and activin receptor genes are expressed in the prostate.

**EXAMPLE 2**  
**DETECTION OF ACTIVIN/INHIBIN PROTEINS**  
**BY RADIOIMMUNOASSAY (RIA)**

- 5 This Example indicates that activin and inhibin are present in the prostate, and that activin is produced by the prostate itself.

**Methods:**

***Reagents***

- 10 Phenylmethyl sulfonyl fluoride (PMSF), BSA, and Triton were purchased from Sigma (St Louis MO). Deoxycholate, Tween 20, Sodium chloride and EDTA were purchased from BDH/Merck (Australia). SDS was from Biorad.

***Animals***

- 15 Adult male Sprague-Dawley rats were obtained from the Central Animal House at Monash University and injected with 75mg EDS (Ethane dimethane sulphonate)/kg body weight in a mixture of DMSO and water as previously described (Risbridger *et al* 1987). Animals were killed in a CO<sub>2</sub> charged chamber at between 3 and 59 days after the administration of EDS as specified in the experiments. Castration was performed under ether anaesthesia through a  
20 midline abdominal incision. The testes were located and the gubernaculum cut to release the epididymis; the testicular artery was ligated and the testis and epididymis removed. The animals were killed in a CO<sub>2</sub> charged chamber 3 days after castration.

***Preparation of cytosols***

- 25 Prostate glands were immediately excised, placed on ice and weighed. A volume of PBS containing 1mM PMSF was added to the tissue in a ratio of between 1:2-40. The samples were aliquoted and frozen prior to radioimmunoassay.

***Detection of activin degradation***

- 30 In order to determine if residual protease activity in the prostate cytosols was able to degrade activin tracer, aliquots of tracer were incubated at 4°C for 48h in the presence of prostate

cytosol or buffer. The incubates were diluted in nonreducing buffer, boiled for 1 min, microfuged for 30s and applied to 15% SDS-acrylamide gels. The resulting gel was sliced into 1mm fractions and the radioactivity measured; the radioactive profiles for buffer controls were compared to those with prostate cytosol and shown to be identical.

5

#### ***Activin radioimmunoassay***

The method is based on that previously published by Robertson *et al* (1992 Endocrinology 130: 1680-1687) with minor modification.

*Tracer:* human recombinant activin A pool G was iodinated by chloramine T and purified by  
10 gel filtration and dye affinity chromatography and used in 0.5% BSA + 0.1% Triton in PBS.

*Standard:* a human recombinant activin A standard (HAS3) was prepared at concentration of 88 ng/ml and serially diluted in PBS + 0.5% BSA.

*Antiserum:* an ovine antiserum which has been previously used for radioimmunoassay was raised to a recombinant B<sub>A</sub> subunit fusion protein and human recombinant activin A and used  
15 at a final dilution of 1:120 000 in CTS reagent (0.125M deoxycholate, 5% Tween 20 and 4% SDS). Limited cross reaction of the antiserum with bovine 31 KDa inhibin and human recombinant 34KDa inhibin, TGFβ1, MIS and follistatin < 3.3% has been previously reported (Robertson *et al* 1992).

#### ***20 Conditions of assay***

A method was devised using delayed tracer addition conditions over 4 days at 4°C. A donkey anti-sheep serum (PBS + 0.5% BSA and 0.01M EDTA) was used to separate bound from free activin tracer.

#### ***25 Inhibin radioimmunoassay***

Samples were assayed for inhibin using antiserum 1989 and the procedure published in Robertson *et al* (1988 Mol Cell Endo 58: 1-8)

## **Results**

### **Activin/inhibin radioimmunoassay**

The radioactive profile obtained following incubation of tracer with buffer or prostate cytosol was identical as shown in Figure 2a,b: non specific degradation of the tracer did not occur  
5 under these conditions.

The prostate cytosol contained detectable levels of immunoactive activin, which diluted in linear and parallel to that of an activin standard - human recombinant standard 3 (Has3). (Figure 3). Inhibin immunoactivity was also detected in these examples.

10

### **EDS studies**

The effect of EDS treatment on prostate weight is shown in Table 2 and confirms previous observations from the inventors' laboratory. Note that a significant decrease in prostate weight was observed in these studies, but that prostate weight had returned to control levels  
15 by day 30.

The levels of activin in prostate cytosols obtained after EDS treatment are shown in Table 2. The data are recorded as ng/organ and ng/g prostate tissue. There is a significant decrease in activin in the prostate and per unit mass of tissue, indicating that activin levels are responsive  
20 to androgen withdrawal. The implication of the changes in concentration of activin may have yet to be determined.

The levels of inhibin are not significantly changed within 3 days of EDS when expressed as levels per unit mass tissue, but are increased thereafter; these data suggest that inhibin is  
25 responsive to androgen withdrawal. Alternatively, as the inhibin RIA detects the pro  $\alpha_c$  fragment of inhibin  $\alpha$ -subunit, the RIA may be measuring an alteration in immunoactive inhibin forms which are responsive to androgens. (Table 2)

### **Castration Studies**

30 The effect of castration on prostate weight has been previously documented but it is noted here that there is a significant drop in prostate weight 3 days after castration. Activin levels

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do not fall significantly within 3 days after castration, and the concentration in the tissue is elevated (Table 3). Changes to inhibin levels are also shown in Table 3.

***Measurement in human seminal plasma samples.***

- 5 Human seminal plasma samples were obtained from semen donors attending the Andrology Clinic Monash Medical Clinic. Samples were diluted and RIA activin levels measured as described, seminal fluid samples did not degrade activin tracer in this assay. Patient samples were also obtained from men undergoing reversal of vasectomy, pre and post-operatively. The results show that the levels of activin in all three groups are not significantly different.
- 10 These data are consistent with the hypothesis that activin is produced in the prostate and seminal vesicles and the levels measured in seminal fluid are not testicular in origin.

**EXAMPLE 3**

**TISSUE COLLECTION**

- 15 Tissues for immunolocalisation were obtained from archival needle biopsy material from 14 men who received no androgen therapy. At least five sections from each biopsy were used for immunochemistry, with antibodies of defined specificity as described below.
- 20 Benign prostatic hyperplasia was confirmed by histologic examination conducted at Melbourne Pathology (Melbourne, Australia).

Ten patients, age range 49-88, who had received no form of androgen therapy, underwent trans-urethral resection of the prostate (TURP) for symptoms of outflow obstruction.

- 25 Following informed consent, and in accordance with procedures and processes required by the Standing Committee for Ethics at Monash University, prostate needle biopsy tissues were collected at surgery under sterile conditions. The specimens were wrapped in sterile foil and snap frozen in liquid nitrogen, before storage at -70°C. Pathological examination of tissues taken at surgery, confirmed benign prostatic hyperplasia.

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Prostate tissues were obtained from a total of 28 patients, which were grouped according to diagnosis into three groups with BPH, basal cell hyperplasia or prostate cancer. Needle biopsies were obtained from 16 patients with BPH, 2 patients with basal cell hyperplasia and 12 patients with prostate cancer (each having a Gleason score grading between seven and ten). None of the patients had received any form of androgen therapy. Two patients with basal cell hyperplasia were identified by histological examinations and diagnosis. The tissues were fixed in 10% buffered formalin and processed in paraffin.

Three micron sections were cut for immunohistochemistry or *in situ* hybridisation as described below.

#### EXAMPLE 4 ANTIBODIES

15 An antibody to human  $\alpha$ -inhibin was purchased from Serotec (UK) and has previously been used and shown to be specific for the localisation of inhibin  $\alpha$ -subunit immunoreactivity (Vliegen *et al.*, 1993).

Antibody (AS #64) was raised against a human  $\beta_A$  subunit fusion protein and human recombinant activin A in sheep. This antibody has been used for the radioimmunoassay of activin A and has no cross reactivity with Mullerian Inhibiting Substance, TGF  $\beta$  and <3.3% cross reactivity with human recombinant inhibin A (Robertson *et al.*, 1992). The radioimmunoassay using AS #64 has been previously used to purify dimeric activin A to homogeneity from ovine amniotic fluid (de Kretser *et al.*, 1994) and for the detection of 25 activin A in biological fluids and samples (McFarlane *et al.*, 1996). This antibody detects both the monomeric and dimeric forms of activin A, and the cross reactivity of the AS #64 with monomeric  $\beta_A$  in the radioimmunoassay was estimated to be 17% (Robertson *et al.*, 1992). To test the specificity of the antibody staining, non-immune serum was used as a control, or the antiserum was preabsorbed with human recombinant activin  $\alpha$  30 Preabsorption of the antisera was achieved by incubating 5 $\mu$ l of undiluted antisera with 1

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$\mu\text{g}$  antigen, either human recombinant activin A or inhibin A, in PBS (200 $\mu\text{l}$ ) at 4°C overnight. The mixture was diluted to a total volume of 1 ml with PBS and centrifuged at 12,000 rpm, and the supernatant decanted and used accordingly.

5 A polyclonal rabbit antibody to inhibin  $\beta_A$  was obtained from Dr W. Vale, of the Salk Institute. A mouse monoclonal antibody to  $\beta_B$  subunit was kindly provided by Dr J. Mather, Genentech (San Francisco, USA). Both have been previously used in the detection of  $\beta$  subunit proteins in ovarian tumour tissue (Gurusinghe *et al.*, 1995).

10 The follistatin antisera, AS #202, was raised in an intact adult male New Zealand rabbit to purified bovine 39 kDa follistatin, and showed <0.5% cross reactivity to bovine inhibin A and bovine activin A (Klein *et al.*, 1991).

An antibody to smooth muscle actin was purchased from Dako Corporation.

15

The polyclonal antibody # $\alpha 41$  was produced by immunisation against recombinant bovine  $\alpha C$  inhibin subunit fusion protein, the sheep was boosted with human  $\alpha C$  inhibin subunit fusion protein and human recombinant inhibin  $\alpha$ . This antibody was used for the detection of the  $\alpha C$  inhibin subunit and has been used previously to measure  $\alpha$ -inhibin levels in

20 serum from normal and postmenopausal women using immunofluorometric assay. A polyclonal antibody # $\alpha 320$  was directed to a fragment (amino acid 1-26) of the fusion protein bovine  $\alpha N$  subunit and used to detect the  $\alpha N$  subunit. Immunostaining for cytokeratin was performed using the monoclonal antibody NCL-LP34 obtained from Novacastra Laboratories (Newcastle Upon Tyne, UK).

25

Additional antibodies used for the detection of the  $\alpha$ -subunit protein immunoreactivity included  $\alpha$  Groome (Serotec) and  $\alpha$  Salk (kindly provided by Professor Vale and Dr J Vaughan).

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## EXAMPLE 5

### IMMUNOSTAINING

After dewaxing, human prostate sections were rehydrated and placed in antigen retrieval solution (Dako, CA, USA) for 20 minutes in a water bath at 85°C. The slides were then washed in PBS and preincubated in CAS block for 30 minutes. Activin A was immunolocalised using AS #64 at a dilution of 1:200, or  $\beta_A$  monoclonal (1:100) and incubated overnight at room temperature. Activin B was localised using the  $\beta_B$  monoclonal antibody (1:100), as was follistatin, using the AS #202 (1:100). Controls were incubated with antiserum preabsorbed with human recombinant activin A (1  $\mu$ g/ml), or a mixture of bovine follistatins (35-, 39-, and 45 kDa) purified from follicular fluid (1  $\mu$ g/ml) or with normal rabbit serum. After overnight incubation, the sections were washed in PBS and incubated with biotinylated rabbit antisheep IgG (activin A), sheep antirabbit ( $\beta_A$  subunit), rabbit antimouse IgG (monoclonal  $\beta_B$ ) or biotinylated goat antirabbit sera (follistatin) (Vector Laboratories, California, USA, 1:200) for one hour. Actin staining was localised using the actin antibody (1:50) for 1 hour. Sections were washed 3 times with PBS (0.01M phosphate buffered phosphate; pH 7.4) and then incubated with rabbit anti-mouse IgG (1:200) for 1 hour. After 2 washes in Tris buffer (0.1M Tris-HCl; pH 8), the sections were incubated in Extravidin Alkaline Phosphatase (Sigma, St Louis, MO, USA) (1:100) for one hour. The New Fuchsin Substrate Kit (Biogenex, CA, USA) was used for the demonstration of Alkaline phosphatase. After colour development, the sections were washed in distilled water, counterstained in haematoxylin, dehydrated, cleansed in xylene, mounted in DPX and analysed by light microscopy.

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## EXAMPLE 6

### IMMUNOHISTOCHEMISTRY

Sections were dewaxed, rehydrated and placed in Target Retrieval solution (Dako, Carpinteria, CA); antigenic sites were exposed by heating at 70°C for 7 minutes. After washing in 0.01M phosphate buffered saline (PBS; 10 mM PO<sub>4</sub>, 154 mM NaCl, pH 7.4), endogenous peroxidase was blocked by 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes. Sections were incubated with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 10 minutes and then blocked with 1:1 mixture of CAS block (Zymed, San Francisco, CA) and 10% normal rabbit serum at room temperature for 20 minutes.

Inhibin was localised using the  $\alpha$ C polyclonal antibody (1.6 $\mu$ g/ml) and the  $\alpha$ N polyclonal antibody 1.9 $\mu$ g/ml). Basal cells were localised using cytokeratin monoclonal antibody (1:100). All antibodies were incubated at 4°C overnight. Controls were incubated with sheep (inhibin) or mouse (cytokeratin) IgG at matched dilution or protein or protein concentration. After overnight incubation the sections were washed in PBS and incubated with biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA; inhibin) or biotinylated rabbit-antimouse IgG (Dako; cytokeratin) for 60 minutes. The secondary antibody was removed and Vectastain Elite ABC Kit (Vector Laboratories) added for 60 minutes. Following further washes with PBS, peroxidase activity was detected using Liquid 3,3' diaminobenzidine tetrahydrochloride (DAB) Substrate Kit (Zymed). The reaction with Mayers' Haematoxylin (Sigma Diagnostics, St Louis, MO) and Scotts water, dehydrated and coverslipped with DPX (BDH, Poole, England).

## EXAMPLE 7

### RT-PCR

#### *Poly A+ RNA Extraction from Human Prostate Needle Biopsy Tissue*

Poly A+ RNA was extracted directly from the tissues using the Dynabeads™ protocol (Dynal, Oslo, Norway). The poly A+ RNA was eluted in 50 $\mu$ l of sterile DEPC-treated

water and stored at -20°C until used.

### ***Oligonucleotide Primer Design***

Oligonucleotide primers for the  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits were designed from human cDNA sequence data obtained from Genbank (access. #M32755 [27], #X57578 [28] and #M13437 [29]). The oligonucleotide primers were designed to span the single intron, and yield products of 169bp, 336bp and 500bp respectively.  $\beta_C$  primers (Schmitt *et al.*, 1996) are believed to span an intron, based on the homology between the  $\beta_A$  and  $\beta_B$  subunit members, and yield a product of 290bp.

10

The PCR primers used for detecting ActRII were designed from the mouse sequence data (Mathews *et al.*, 1991) and span the extracellular and transmembrane domains, to yield a product of 510bp. Follistatin primers (Meinhardt *et al.*) were designed to span exons 5 and 6 of the human follistatin sequence and yield two products of 207bp and 470bp corresponding to FS 315 and FS 288, respectively. The deduced precursor sequences bear no homology with the  $\alpha$ ,  $\beta_A$  and  $\beta_B$  chains. PCR conditions and primer sequences are outlined in Table 4.

15

### ***Reverse Transcription (RT)***

Reverse transcription for all mRNAs was carried out using 20 $\mu$ l of A+ RNA, denatured at 65°C for 5 min, and mixed with 30 U reverse transcriptase (Promega Biotec, Madison, WI, USA), 40 U RNasin (Promega Biotec, Madison, WI, USA), 15 pmol Oligo (dT)<sub>15</sub> primer (Promega Biotec, Madison, WI, USA), 1 mM of each dATP, dTTP, dCTP, dGTP (Promega Biotec, Madison, WI, USA) to a final volume of 50 $\mu$ l. The solution was incubated at 42°C for 2 hours, heated to 95°C for 2 min and cooled rapidly on ice.

25

### ***Polymerase Chain Reaction (PCR)***

The PCR was performed in a Perkin Elmer Cetus DNA Thermal Cycler as previously described by Saiki *et al.*, (Saiki *et al.*, 1985). Briefly, 10  $\mu$ l of the RT mixture was added to 30 pmol of each primer, and 1 U of Ampli Taq DNA polymerase (Roche Molecular

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Systems Inc., Branchburg, New Jersey), in 1 x PCR buffer (Roche Molecular Systems Inc., Branchburg, New Jersey) to a final volume of 50  $\mu$ l. PCR products were analysed by Nusieve GTG agarose gel electrophoresis in 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA).

5

Samples from human prostate tissues were Southern blotted and sequence identity confirmed.

## EXAMPLE 8

10

### SOUTHERN BLOTTING RT-PCR PRODUCTS

#### *Probe Labelling*

Probes were labelled either with DIG or with  $^{32}$ [P] for Southern analyses. Probes for ActRII,  $\beta_A$  and  $\beta_C$  were derived from sequenced PCR products, and were labelled with  
15 DIG. Briefly, 25 ng of denatured probe was mixed with 1 x hexanucleotide mix (Boehringer Mannheim GmbH Biochemica, Germany), 1 x dNTP labelling mix (Boehringer Mannheim GmbH Biochemica, Germany), 2  $\mu$ l 0.25 mM DIG-dUTP (pH 6.5), and 5U of Klenow enzyme (Promega Biotec, Madison, WI, USA) to a final volume of 20 $\mu$ l. The mixture was incubated at 37°C for 1 hour. 20mM of EDTA was added to  
20 stop the reaction, before storing the probe at -20°C.

The FS (Michel *et al.*, 1990)  $\alpha$  Najmabadi *et al.*, 1993)  $\beta_A$  (Esch *et al.*, 1987) and  $\beta_B$  (Stewart *et al.*, 1986) probes were labelled with  $^{32}$ [P]. Briefly 25 ng of denatured probe was mixed with 1 x hexanucleotide mix (Boehringer Mannheim GmbH Biochemica,  
25 Germany), 1 mM of each dATP, 1 mM dGTP, 1 mM dTTP (Promega Biotec, Madison, WI, USA), 4 mCi of  $^{32}$ [P] dCTP (DuPont, NEN Research Products, Boston, MA, USA) and 10 U of Klenow enzyme (Promega Biotec, Madison, WI, USA) to a final volume of 20  $\mu$ l, and incubated overnight at room temperature. The probe was precipitated after the  
30 addition of 3  $\mu$ g herring sperm DNA (Promega Biotec, Madison, WI, USA), 0.0 M sodium perchlorate, 0.4 vol isopropanol in a final volume of 165  $\mu$ l and centrifuged at

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13,000g for 5 min. Labelling efficiency was determined by scintillation counting (1900 TR Liquid Scintillation Analyser, Packard Instrument Co., Ulgersmaweg, The Netherlands).

### 5 *DIG Southern Blot Analysis of RT-PCR Products*

Southern blot analysis of RT-PCR products was carried out as follows. Membranes were pre-hybridised for 1 hour in prehybridisation buffer (DIG {5x SSC, 0.1% N-laurolysarcosine, 0.02% sodium dodecyl sulfate, 1% Blocking Reagent (Boehringer Mannheim GmbH Biochemica, Germany)} and  $^{32}$ [P] {Rapid Hyb (Amersham Life Science, Buckinghamshire, UK)}), before addition of denatured probe. Hybridisation was carried out for 2 hrs at 65°C, before washing for 15 min, twice with 2 x SSC + 0.1% SDS, and twice 0.5 x SSC + 0.1% DIG labelled probes were detected using anti-DIG antibody conjugated to alkaline phosphatase, followed colorimetric analysis as per manufacturers directions.  $^{32}$ [P] labelled probes were detected by autoradiography using XOMAT AR film (Eastman Kodak Co., NY, USA) with intensifying screens at -75°C.

## EXAMPLE 9

### IN SITU HYBRIDISATION

#### 20 *Probe Synthesis*

Digoxigenin (Dig) labelled riboprobes were prepared using the method outlined in the Boehringer Mannheim riboprobe labelling kit. Rat and human inhibin  $\alpha$ -subunit share an 82% homology and riboprobes to both rat and human sequences were used in this study.

25 Dig antisense and sense cRNA probes (gift from Dr. Moira O'Bryan, Institute of Reproduction and Development, Monash University, Melbourne, Australia) were synthesised from a ~400bp partial rat  $\alpha$ -inhibin subunit cDNA cloned into pGem 4Z (Esch *et al.*, 1987). Antisense probes were transcribed from *Eco*RI linearised plasmids with T7 RNA polymerase and sense cRNA was generated from *Hind*III linearised  
30 plasmids with SP6 RNA polymerase. The amount of Dig-labelled RNA was determined

by comparison to a Dig-labelled RNA control using dot blot analysis.

An  $\approx$  400bp *Pst*I/*Pvu*II fragment of the human inhibin  $\alpha$ -ubunit cDNA (gift from Biotech, Roseville, NSW, Australia) was subcloned into pGEM 4z. The cDNA corresponds to  
5 positions 702-1115 of the published human inhibin  $\alpha$ -subunit nucleic acid sequence  
(Mason *et al.*, 1986). Antisense probes were synthesised by linearising the plasmids with  
*Hind*III and transcribed with SP6 RNA polymerase. Sense probes were obtained after  
linearising with *Eco*RI and transcribed with T7.

- 10 After dewaxing, sections were washed in 1xPBS (2x5min) and treated with proteinase K  
(20 $\mu$ g/ml) for 30 min at 37°C. Following digestion sections were washed in PBS  
containing 0.2% glycine for 5 min followed by 5 min fixation in 4% Paraformaldehyde.  
Sections were then washed in PBS 2x5 min, equilibrated for 2 min in 0.1M  
triethanolamine and acetylated in 0.25% acetic anhydride in Triethanolamine for 5 min.
- 15 After rinsing in PBS prehybridisation was conducted at 42°C for 60 min in hybridisation  
buffer which contained 50% formamide, 10% dextran sulphate, 1x Denhardts, 5x SSC  
(sodium citrate, 1x = 0.15M NaCl, 0.015 M Na citrate), 45mM phosphate buffer,  
hsDNA (200 $\mu$ g/ml; Progema, WI, U.S.A.) and tRNA (500 $\mu$ g/ml, Sigman, Mo, U.S.A.).  
Riboprobe was diluted in hybridisation buffer to a concentration of 200-1000 $\mu$ g/ml and  
20 denatured at 65°C for 10 min to remove secondary structures. Slides were then incubated  
at 80°C for 10 min and hybridisation was performed under coverslips in a humidified box  
at 42°C overnight.

Following hybridisation coverslips were removed in 4xSSC and slides were then washed  
25 2x5 min in 2xSSC. An Rnase A digestion (20 $\mu$ g/ml) was performed at 37°C for 30 min  
followed by SSC washes of increasing stringency, 2x5 min in 1xSSC, 1x20 min in  
0.5xSSC at 42°C. The tissues were briefly rinsed in 0.1M Maleic acid/0.15MNaCl (ph  
7.5) and non-specific binding was removed with a Blocking Buffer containing 1% Skim  
milk powder in 0.1M Maleic acid/0.15MNaCl (ph 7.5) for 30 min at RT. Slides were  
30 then incubated in Casblock (Zymed) for 20 min at RT. An anti-digoxigenin alkaline

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phosphate conjugate antibody (Boehringer) was diluted 1:1000 in Blocking Buffer and sections were incubated overnight at 4°C. After washing 3x10 min in 0.1M Maleic acid/0.15M NaCl, immunoreactivity was detected with NBT/BCIP substrate (NBT/BCIP) one step; Pierce, Rockford, IL, USA). After appropriate colour development (1-20 hours) the reaction was halted by immersion in water.

### EXAMPLE 10

#### IMMUNOLOCALISATION STUDIES

10 The pattern of localisation of inhibin  $\alpha$ ,  $\beta_A$ , and  $\beta_B$  subunits is shown in Figure 4, and was determined using specific monoclonal and polyclonal antibodies to the  $\alpha$  and  $\beta$  subunit proteins. As shown in Figure 4 A and B, no  $\alpha$  immunoreactivity could be detected in BPH tissues, although  $\alpha$  immunoreactivity was readily detectable in positive control sections of human ovarian benign cystadenoma (Fig 4C).  $\beta_A$  subunit reactivity was predominantly  
15 localised to the epithelial tissues (Fig 4D) and it was noted that the staining intensity was variable within, and between, the glandular structures in the same sections (Fig 4D, E). No immunoreactivity was present in the control sections (Fig 4F). Weak, but detectable, immunoreactivity for the  $\beta_B$  subunit was localised to the epithelium in BPH tissues (Fig 4G). There was variability in the intensity of staining for  $\beta_B$  subunit as shown in (Fig 4H,  
20 I). Collectively these data demonstrate that the  $\beta_A$  and  $\beta_B$  but not  $\alpha$ , subunit proteins can be detected by immunolocalisation in BPH tissues, using these antibodies.

### EXAMPLE 11

#### INHIBIN/ACTIVIN $\alpha$ , $\beta$ SUBUNIT, FOLLISTATIN AND ActRII mRNA 25 EXPRESSION

As limited amounts of tissue were obtained, two groups of patient tissues were used for analysis of mRNA expression. Patient samples a-e, were used to determine the presence of the activin receptor, ActRII, inhibin  $\beta_A$  and the putative activin  $\beta_C$  mRNA; patent samples  
30 f-j were used to determine follistatin, and inhibin  $\alpha$  and  $\beta_B$  subunit mRNA expression.

Total RNA from rat testes (Figure 5, Lane t) and rat prostate (Figure 5, Lane p) were used as positive controls for each of the primer pairs.

***mRNA expression of ActRII,  $\beta_A$  and  $\beta_C$  in patient samples a-e***

5 The data in Figure 5A-C demonstrate the mRNA for the activin receptor (ActRII), inhibin  $\beta_A$  subunit, and the putative  $\beta_C$  subunit are expressed in human prostate tissue samples a-e. Figure 5A shows the detection of ActRII mRNA in all five biopsy samples from patients a-e (Lanes a-e respectively), and demonstrated the integrity of the extracted mRNA. Inhibin  $\beta_A$  subunit mRNA expression was detected by Southern analysis in three of the  
10 five patient samples (Figure 5B, lanes c, d, e), suggesting variability in  $\beta_A$  mRNA expression. Whereas the putative  $\beta_C$  subunit mRNA was detected in all of the patent samples (Figure 5C, Lanes a-e).

***mRNA expression of follistatin and inhibin  $\alpha$  and  $\beta_B$  subunit in patient samples f-j***

15 mRNA for the activin binding protein, FS 288 was detected in only two of the five biopsy samples (Figure 5D, Lane i and j respectively); however the alternate splice variant, FS 315, was readily detected in all five patent samples (Figure 5D, Lanes f-j respectively). These results confirm the integrity of the mRNA. However, inhibin  $\beta_B$  mRNA expression was weakly detected in two of the five biopsies (Figure 5E, Lanes f and g), thus the  
20 ability to detect inhibin  $\beta_B$  subunit mRNA in the human prostate was variable between the patent samples. Inhibin  $\alpha$ -subunit mRNA expression was also detected in four biopsy samples f, g, i and j (Figure 5F Lanes f, g, i & j respectively)

**EXAMPLE 12**

**BPH TISSUES**

25 In the glandular epithelial tissue from patients with BPH, basal cells were localised using a specific cytokeratin monoclonal antibody as shown in Figure 6A. No immunoreactivity was observed in the control sections (Figure 6B). Positive immunoreactivity was localised  
30 to epithelial cells in tissue sections from 11 patients with BPH using specific antibodies to

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$\alpha$ C and/or  $\alpha$ N inhibin subunit proteins. As shown in Figure 6C, inhibin  $\alpha$ C subunit was readily detected in basal cells (6 of 8 patient tissues) and there was variable immunoreactivity in the luminal secretory cells (2 of 8 patient tissues). No immunoreactivity was present in the control section (6 of 6 patient tissues; Figure 6E).

- 5 Positive immunoreactivity for the inhibin  $\alpha$ N subunit was localised to both the secretory epithelium and basal cells (Figure 6D). No immunoreactivity was detected in the control section (Figure 6F). No immunoreactivity was localised to any patient tissue using the Groome or Salk antibodies raised to the  $\alpha$ -subunit of inhibin.
- 10 Using *in situ* hybridisation with both rat and human DIG labelled riboprobes, mRNA for inhibin  $\alpha$ -subunit was localised to the epithelial basal cells (Figure 6G) in 5 patients. In 1 out of 5 patients  $\alpha$  mRNA was localised to basal and secretory epithelial cells (Figure 6H). The sense probe displayed no staining (Figure 6I and J). A summary of these results is presented in Table 5A.

15

### EXAMPLE 13 BASAL CELL HYPERPLASIA

- Tissue sections obtained from two patients with basal cell hyperplasia were used to detect
- 20 inhibin  $\alpha$ -subunit gene expression and protein localisation was determined. Identification of regions of basal cell hyperplasia was confirmed using a cytokeratin antibody as shown in Figure 7A. No immunoreactivity was localised in the control section (Figure 7B).  $\alpha$ C and  $\alpha$ N inhibin subunit protein immunoreactivity was also localised to these regions of the tissue sections and confirmed that inhibin proteins are localised to basal cells as shown in
- 25 Figure 7C and E, respectively. No immunoreactivity was detected in the control sections (Figure 7D and F). The expression of inhibin  $\alpha$ -subunit mRNA in basal cell hyperplasia was confirmed in one patient using *in situ* hybridisation (Figure 7G); no localisation was detected using the corresponding sense labelled riboprobe (Figure 7H).

## EXAMPLE 14

### PROSTATE CANCER

In 12 patients with poorly differentiated prostate cancer, the localisation of  $\alpha$ C protein (9 patients),  $\alpha$ N protein (6 of 12 patients) and  $\alpha$ -inhibin mRNA (8 of 12 patients) was determined and compared in malignant and adjacent non-malignant regions of the tissues. As observed in tissue from patients with BPH, the  $\alpha$ C protein was predominantly localised to the basal cells of non-malignant regions of tissue sections in 8 of 11 patient tissues (Figure 8A) and to basal and secretory cells in 3 of 11 patient tissues. In the adjacent poorly differentiated tumour tissue no positive immunoreactivity was observed (Figure 8B). Similarly, the pattern of staining of the  $\alpha$ N to the basal cells of non-malignant regions of tissue sections in 8 of 11 patient tissues (Figure 8A) and to basal and secretory cells in 3 of 11 patient tissues. In the adjacent poorly differentiated tumour tissue no positive immunoreactivity was observed (Figure 8B). Similarly, the pattern of staining of the  $\alpha$ N protein was predominantly localised to the basal and epithelial cells in the non-malignant region of tissue sections from men with advanced stage cancer of the prostate (Figure 8C): no immunoreactivity was observed in the adjacent tumour tissue (Figure 8D). The control for both the malignant and non-malignant regions displayed no positive staining (Figure 8E and F, respectively).

20

*In situ* hybridisation was performed using tissue from 8 patients with histological grade 4/5 prostate cancer and confirmed the pattern of protein inhibin localisation. Hence,  $\alpha$ -subunit gene expression was detected in basal cells in 7 of 8 patients in non-malignant regions and in both basal and secretory cells in some patients (Figure 8G). Malignant tumour cells in adjacent regions of the same patient biopsies did not display any  $\alpha$ -subunit gene expression (Figure 8H). No staining was observed with the inhibin  $\alpha$  sense riboprobe (Figure 8I and J). The results for the non-malignant regions of the patient tissue are summarised in Table 5C.

30

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Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in  
5 this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

**Table 2** The effect of EDS, 3 and 14 days after administration, on prostate weight and the levels of ir-inhibin and ir-activin in the prostate, expressed as ng/g tissue and ng/organ.

Values are means  $\pm$  S.D., n=5

	<b>3-Day control (DMSO)</b>	<b>3-Day EDS</b>	<b>14-Day control (DMSO)</b>	<b>14-Day EDS</b>
Prostate weight	0.56 $\pm$ 0.15	0.38 $\pm$ 0.05	0.49 $\pm$ 0.07	0.07 $\pm$ 0.02*
<b>ir-Inhibin</b>				
ng/g tissue	19.1 $\pm$ 2.2	22.1 $\pm$ 3.4	15.7 $\pm$ 3.4	13.9 $\pm$ 4.4
ng/organ	11.1 $\pm$ 2.8	8.43 $\pm$ 1.9	7.6 $\pm$ 1.5	1.0 $\pm$ 0.4*
<b>ir-Activin</b>				
ng/g tissue	240 $\pm$ 156	277 $\pm$ 203	319 $\pm$ 224	264 $\pm$ 137
ng/organ	136 $\pm$ 86	101 $\pm$ 65	168 $\pm$ 145	17 $\pm$ 8*

\* $P < 0.01$  vs control (data analysed using ANOVA and Student-Newman-Keuls multiple range test).

**Table 3** The levels of ir-inhibin and ir-activin measured by radioimmunoassay in normal adult rats testis and in normal and 3-day castrate rat prostate cytosols.

Values are means  $\pm$  S.D., n=5 tissues

	Normal adult testis	Normal adult prostate	Prostate from 3-day castrate
Organ weight	1.596 $\pm$ 0.093	0.595 $\pm$ 0.070	0.294 $\pm$ 0.102*
<b>ir-Inhibin</b>			
ng/g tissue	9.10 $\pm$ 1.85	11.38 $\pm$ 3.03	15.91 $\pm$ 1.88
ng/organ	14.47 $\pm$ 2.76	6.89 $\pm$ 2.30	4.74 $\pm$ 1.95
<b>ir-Activin</b>			
ng/g tissue	162 $\pm$ 32	376 $\pm$ 59	574 $\pm$ 76
ng/organ	258 $\pm$ 50	223 $\pm$ 44	168 $\pm$ 61

\* $P < 0.01$  vs control (data analysed using ANOVA and Student-Newman-Keuls multiple range test).

TABLE 4. Sequence and PCR Conditions for Oligonucleotide Primers

Sequence	5' to 3' sequence	SEQ ID NO	Cycle conditions			Cycles
ActRII	CGG GAT CCA ACT GCT ATG ACA G	9	95°C	56°C	72°C	35
	GGA ATT CGC ACC AAR GAA CTG	10				
Follistatin	TTC CCT CTG TGA TGA GCT GTG	11	95°C	60°C	72°C	40
	AGC TGT AGT AGT CCT GGT CTT CAT	12				
$\alpha$	CAT GCA GAC CTC TGA ACC AG	13	95°C	65°C		40
	GTG GCT GCG TAT GTG TTG GGA TG	14				
$\beta_A$	CTT GAA GAA GAC CCG ATG TCA C	15	95°C	53°C	71°C	40
	AAG AGG ATG GTG ACT TTG GTC	16				
$\beta_B$	GAA ATC ATC AGC TTC GCC GAG AC	17	95°C	57°C	71°C	50
	GAA CTG TTG CCT GCA ACA GAG GTT G	18				
$\beta_C$	ATG ACC TCC TCA TTG CTT CTG GC	19	95°C	53°C	71°C	50
	TTC ACA TTC CAG TTC CCT GTT GTC	20				

**TABLE 5A and 5B.** Summary of the positive expression and localization of inhibin  $\alpha$ -subunit in tissue from men with benign prostatic hyperplasia (BPH) (A), basal cell hyperplasia (BCH) (B), and prostate cancer (PCA) (C)

Patient No.		Immunoreactivity		<i>In Situ</i> ...
		$\alpha_N$	$\alpha_c$	
A.	BPH tissues			
	1	+bs		
	2	+bs		
	3	+bs		+b
	4	+bs	+b	
	5	+bs	+bs	
	6	+bs	+b	
	7		+b	
	8		+b	
	9		+b	
	10		+bs	
	11		+b	
	12			+b
	13			+bs
	14			+b
	15			+b
B.	BCH tissues			
	1	+b	+b	
	2	+b	+b	+b

+, Positive localization of protein or mRNA expression; b, staining in basal cells; s, staining in secretory epithelial cells; \*, nonmalignant regions of tissue were not present in the sections used; only regions of tumor were present, so the localization in adjacent nonmalignant regions could not be described for these two patients. Note that not all patient tissues were used for the localization of  $\alpha_c/\alpha_N$  protein and mRNA expression; most tissues were used for either immunochemistry or *in situ* hybridization.

**TABLE 5C.** Summary of the positive expression and localization of inhibin  $\alpha$ -subunit in tissue from men with benign prostatic hyperplasia (BPH) (A), basal cell hyperplasia (BCH) (B), and prostate cancer (PCA) (C)

	Patient No.	Immunoreactivity		<i>In Situ</i>
		$\alpha_N$	$\alpha_C$	
C.	1		+b	+b
	2	+bs	+b	+bs
	3		+b	+b
	4	*	*	+bs
	5	+bs	+b	+bs
	6	+bs	+b	
	7	+bs	+bs	+bs
	8	+bs	+bs	
	9	+bs	+b	*
	10		+bs	
	11		+b	+bs
	12		+b	+b

+, Positive localization of protein or mRNA expression; b, staining in basal cells; s, staining in secretory epithelial cells; \*, nonmalignant regions of tissue were not present in the sections used; only regions of tumor were present, so the localization in adjacent nonmalignant regions could not be described for these two patients. Note that not all patient tissues were used for the localization of  $\alpha_C/\alpha_N$  protein and mRNA expression; most tissues were used for either immunochemistry or *in situ* hybridization.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: MONASH UNIVERSITY
- (ii) TITLE OF INVENTION: MODULATION OF CELL GROWTH AND METHODS RELATING THERETO
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: DAVIES COLLISON CAVE
  - (B) STREET: 1 LITTLE COLLINS STREET
  - (C) CITY: MELBOURNE
  - (D) STATE: VICTORIA
  - (E) COUNTRY: AUSTRALIA
  - (F) ZIP: 3000
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: INTERNATIONAL APPLICATION
  - (B) FILING DATE: 23-APR-1998
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- (vii) PRIOR APPLICATION DATA:
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  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: SLATTERY, JOHN M.
  - (B) REFERENCE/DOCKET NUMBER: JMS/TDO/DK
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: +61 3 9254 2777
  - (B) TELEFAX: +61 3 9254 2770
  - (C) TELEX: AA 31787

- 51 -

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGCCCAGCTC CTGGAAGGAG AT

22

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCAGCCCAGC TGTGGTTCCA C

21

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAATTCGCA CCAATGAACT G

21

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGGATCCAA CTGCTATGAC AGG

23

- 52 -

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAGGACAATG TGGCTTCGGG TGG

23

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCCAGCACC GCGGTGAG

18

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGGCTGTGA AGATCTCC

18

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGCTCATCA CAGCCTT

17

- 53 -

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGATCCAA CTGCTATGAC AG

22

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGAATTCGCA CCAARGAACT G

21

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTCCCTCTGT GATGAGCTGT G

21

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCTGTAGTA GTCCTGGTCT TCAT

24

- 54 -

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CATGCAGACC TCTGAACCAG

20

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGGCTGCGT ATGTGTTGGG ATG

23

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTTGAAGAAG ACCCGATGTC AC

22

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAGAAGATGG TGACTTTGGT C

21

- 55 -

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAAATCATCA GCTTCGCCGA GAC

23

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAACTGTTGC CTGCAACAGA GGTTC

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## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATGACCTCCT CATTGCTTCT GGC

23

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCACATTCC AGTTCCTGT TGTC

24

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